Expression of 1L-Myoinositol-1-Phosphate Synthase in Organelles[1]

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We have studied the expression of 1L-myoinositol-1-phosphate synthase (MIPS; EC 5.5.1.4) in developing organs of *Phaseolus vulgaris* to define genetic controls that spatially regulate inositol phosphate biosynthesis. MIPS, the pivotal biosynthetic enzyme in inositol metabolism, is the only enzyme known to catalyze the conversion of glucose 6-phosphate to inositol phosphate. It is found in unicellular and multicellular eukaryotes and has been isolated as a soluble enzyme from both. Thus, it is widely accepted that inositol phosphate biosynthesis is largely restricted to the cytosol. Here, we report findings that suggest the enzyme is also expressed in membrane-bound organelles. Microscopic and biochemical analyses detected MIPS expression in plasma membranes, plastids, mitochondria, endoplasmic reticula, nuclei, and cell walls of bean. To address mechanisms by which the enzyme could be targeted to or through membranes, MIPS genes were analyzed for sorting signals within primary structures and upstream open reading frames that we discovered through our sequence analyses. Comprehensive computer analyses revealed putative transit peptides that are predicted to target the enzyme to different cellular compartments. Reverse transcriptase PCR experiments suggest that these putative targeting peptides are expressed in bean roots and leaves.

Inositol metabolism is essential for the development of plants, animals, and some microorganisms. Metabolites of inositol (a six carbon cyclitol) such as phosphoinositides and phytate function as potent regulators of signal transduction for a wide variety of hormones, growth factors, and neurotransmitters (York et al., 1999; Loewus and Murthy, 2000; Irvine and Schell, 2001). Inositol phosphate, the immediate precursor of free inositol, is synthesized via the internal cyclization of Glc 6-phosphate. The overall reaction mechanism consists of a tightly coupled oxidation and reduction (Sherman et al., 1969; Loewus and Loewus, 1983). 1L-Myoinositol-1-phosphate synthase (MIPS; EC 5.5.1.4) is the only enzyme known to catalyze this reaction. MIPS is found in diverse organisms, both eukaryotic and prokaryotic, suggesting that the pathway for inositol 1-phosphate biosynthesis from Glc 6-phosphate arose early in the evolution of life (Majumder et al., 1997; Bachhawat and Mande, 2000). The properties and generally accepted catalytic mechanisms of the enzyme are similar in all organisms where such assessment has been undertaken (Loewus and Murthy, 2000). Alignment of MIPS amino acid sequences from diverse organisms including Arabidopsis, bean (*Phaseolus vulgaris*), Brewer's yeast (*Saccharomyces cerevisiae*), and *Entamoeba histolytica* revealed remarkable evolutionary conservation of the primary structure (Majumder et al., 1997). Genome sequencing projects have provided additional evidence for this striking conservation with deduced primary structures from organisms such as *Caenorhabditis elegans*, fruitfly (*Drosophila melanogaster*), *Leishmania major*, and *Chlamydomonas reinhardtii*.

Although the essential roles of inositol in many cellular processes including membrane formation, cell wall biogenesis, stress response, and signal transduction have been well documented, less is known of the cellular mechanisms that regulate its complex metabolic flux. To identify spatial and temporal controls that regulate the biosynthesis of inositol phosphate, we have isolated and studied MIPS genes and gene products from Arabidopsis and bean using yeast inositol mutants and yeast MIPS polyclonal antibody (Johnson, 1994; Johnson and Burk, 1995; Johnson and Sussex, 1995; Wang and Johnson, 1995; Johnson and Wang, 1996). Here, we report microscopic and biochemical findings concerning the compartmentalization of MIPS (i.e. inositol phosphate biosynthesis) in organs of bean.

RESULTS

MIPS Expression during Root Development

MIPS expression was monitored during root development using western-blot analyses. Isoforms of the enzyme were identified and partially purified from roots of 8-d-old plants (Fig. 1, A and B). Three of these forms (80, 56, and 33 kD) were previously shown to be temporally and spatially regulated during bean (Johnson and Wang, 1996) and Arabidopsis (Johnson, 1994; Johnson and Sussex, 1995) develop-

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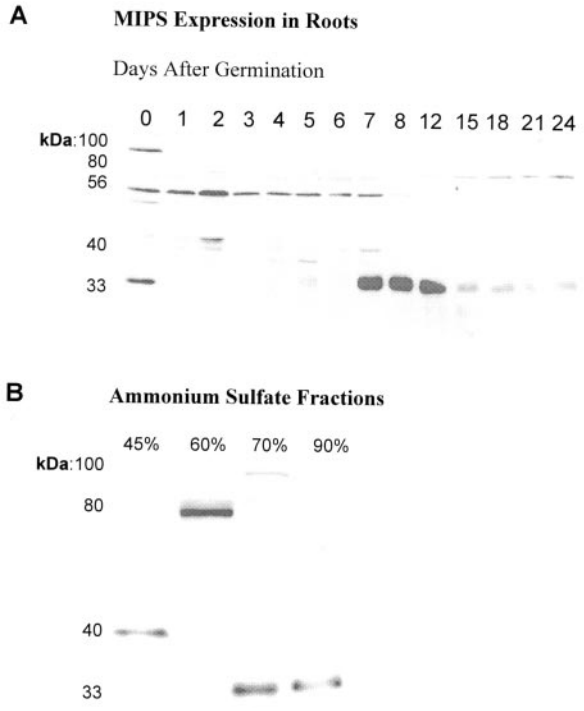

Figure 1. Isoforms of MIPS are differentially expressed during root development. A, Western-blot analyses of bean root proteins during development detected five cross-reacting proteins including the 80-, 56-, and 33-kD proteins previously characterized (Johnson and Wang, 1996). Antigen capture immunoassays (Weiler et al., 1960), ammonium sulfate fractionations, and enzyme assays suggest these proteins are isoforms of MIPS. Each lane contains total proteins (50 μg). B, MIPS was partially purified from roots harvested 8 d after germination. Ammonium sulfate precipitates of 45%, 60%, 70%, and 90% produced 27, 64, 42, and 56 nmol inositol phosphate h^{-1} mg^{-1} protein, respectively.

ment. In plants and in animals, the existence of multiple isoforms of particular enzymes often reflects the number of subcellular compartments in which the same catalytic reaction is required (Gottlieb, 1982). Thus, we hypothesized that the number of MIPS isoforms detected might reflect the distribution of the enzyme to other cellular compartments in addition to the cytosol and chloroplast (Johnson and Wang, 1996). To address this hypothesis, we first conducted immunolocalization studies.

Immunolocalization

Immunohistochemical experiments provided valuable overviews of MIPS expression in cells of bean roots (Fig. 2, A and B) and leaves (Fig. 2, C and D). The enzyme is expressed in intracellular structures and in cell walls (Fig. 2B). In addition, it is highly expressed in the vascular system of leaves (Fig. 2D) where many inositol-containing compounds and other inositol metabolic enzymes have been identified (Gillaspy et al., 1995).

Immunocytochemistry was used to identity the subcellular structures. Micrographs of bean roots (Fig. 3, A and B) detail MIPS expression in plastids, mitochondria, endoplasmic reticula, plasma membranes, cell walls, nuclei, and nucleoli. The specificity of these results was verified by counting the number of gold particles present in each organelle in six samples (grids incubated with primary antibody and goat anti-rabbit 10-nm gold-conjugated secondary antibody, respectively) and in six controls (grids incubated with goat anti-rabbit 10-nm gold-conjugated secondary antibody only; Fig. 3). The difference, as

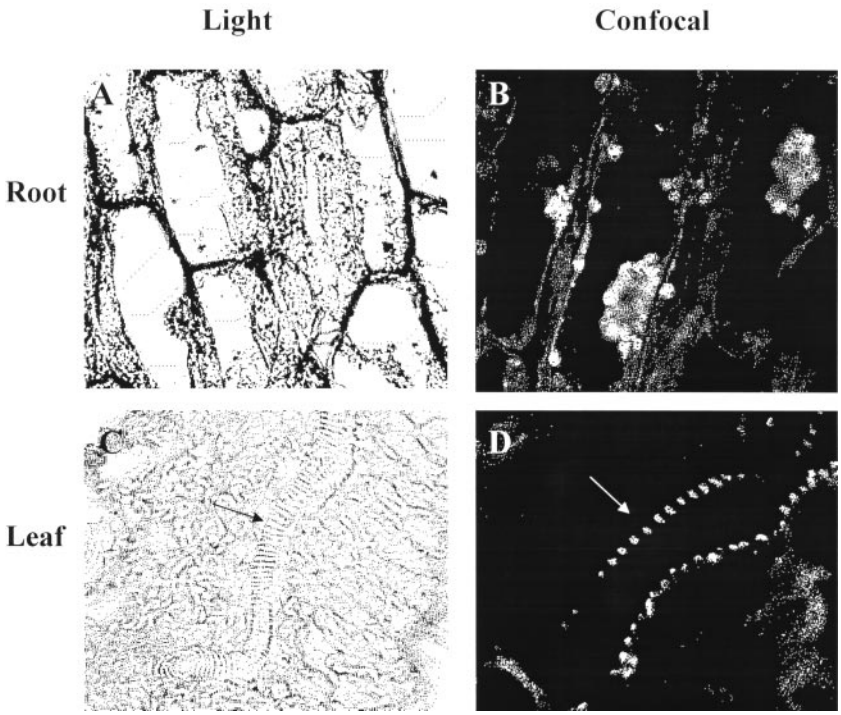

Figure 2. Immunohistochemical studies profiled MIPS expression in organs of bean. Sections of 8-d-old bean roots (A and B) and leaves (C and D) were stained with toluidine blue and incubated with MIPS antibody and fluorescein isothiocyanate-conjugated secondary antibody. Shown are light (A) and confocal (B) micrographs of a longitudinal section of root and light (C) and confocal (D) micrographs of leaf section (arrows show vascular system). Root and leaf light micrographs were magnified 580× and 370×, respectively. Bar = 20 μm. Magnification of confocal micrographs could not be determined with the Nikon PCM 2000 microscope. Controls included unstained sections not treated with primary or secondary antibody to detect autofluorescence and stained sections incubated only with secondary antibody to detect nonspecific interactions.

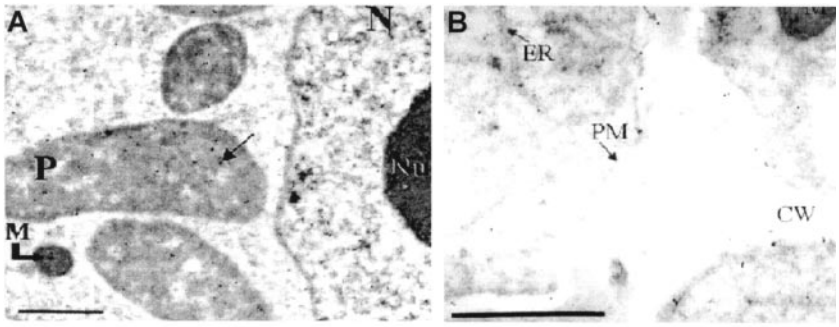

Figure 3. Immunocytochemical analyses identified membrane-bound organelles as subcellular locations of MIPS. Bean root sections were incubated with MIPS primary antibody and goat anti-rabbit 10-nm gold-conjugated secondary antibody and photographed with a Zeiss 10A TEM microscope. Controls were incubated with goat anti-rabbit 10-nm gold-conjugated secondary antibody only. As shown in A and B, gold particles are present in plastids (P), mitochondria (M), the nucleus (N), nucleolus (Nu), endoplasmic reticula (ER), plasma membrane (PM), and the cell wall (CW). Bar = 1 μm.

Immunocytochemical studies identified membrane bound organelles as sites of MIPS in *Phaseolus* roots. The difference between the average number of gold particles present in cells of samples and in cells of controls was shown to be significant. x 15,584.

	Average number of gold particles per cell per					
	Organelle		square micron		micron of membranes	
	sample	control	sample	control	sample	control
Mitochondria	55	16				
Plastids	136	18				
Endoplasmic Reticula					98	7
Plasma Membranes					203	11
Cell Walls					176	5
Cytoplasm			601	55		
Vacuoles			211	96		
Nuclei	132	12				
Nucleoli	117	7				

determined by Tukey's honestly significant difference test (Daniel, 1995), was shown to be significant.

Subcellular Fractionation

To biochemically corroborate the microscopic studies, organelles (mitochondria, plastids, endoplasmic reticula, plasma membranes, and chloroplasts) were isolated from roots and leaves and assayed for purity and enrichment using organelle-specific marker enzymes (Tables I and II). MIPS activity as determined by the end-product method (Chen and Charalampous, 1966) and the rapid colorimetric method (Barnett et al., 1970) was detected in all isolated organelles. Yeast soluble and insoluble (microsomal) fractions were used as positive and negative controls, respectively, because earlier studies indicated that MIPS was found in soluble cellular fractions (Donahue and Henry, 1981; Johnson and Henry, 1989). MIPS expression was detected in both controls.

Computer Analyses

MIPS has a very highly conserved primary structure and has been isolated from numerous organisms, yet such a broad distribution was unsuspected, and no mechanisms for localization to cellular compartments other than cytosol and chloroplasts have been reported (Majumder et al., 1997). We sought a causal explanation from comprehensive computer analyses of four genomic copies of MIPS, one from bean and three from Arabidopsis.

The programs, PSORT (Prediction of Protein Sorting Signals and Localization Sites in Amino Acid

Table I. *Solubilized purified organelles exhibit MIPS activity*

MIPS specific activity was determined in 0.003 mg of organelle protein using periodate oxidation to measure release of inorganic phosphate from inositol phosphate. One unit of MIPS is defined as 1 nmol of inositol phosphate produced per hour. Total activity, Nanomoles of inositol phosphate produced per hour (units $\times 10^{-3}$). Specific activity, Nanomoles of inositol phosphate per hour per 0.003 mg of protein (units/mg $\times 10^{-3}$).

Fraction ([Protein] = 0.003 mg)	Total Activity	Specific Activity
	units	*units mg^{-1}*
Root		
Plastids	43	14.333
Mitochondria	38	12.666
Microsomes	27	9.000
Plasma membranes	64	21.333
Leaf		
Chloroplasts	12	4.000
Mitochondria	40	13.333
Plasma membranes	67	22.333

Table II. *Solubilized purified organelles exhibit MIPS activity*

To assess organelle purity and enrichment, marker enzymes for endoplasmic reticula (antimycin insensitive NADH cytochrome C reductase), mitochondria (cytochrome C oxidase), plastids (nitrite reductase), plasma membranes (vanadate-sensitive Ca^{2+} ATPase), and microbodies (catalase) were assayed using 0.010 mg of protein for each fraction. Contamination between organelles was estimated by comparing the specific activity (S.A.) of the marker enzymes in each fraction with the specific activity of the marker enzyme in its designate organelle.

Fraction ([Protein] = 0.010 mg)	Specific Activity				
	Cytochrome C Reductase	Cytochrome C Oxidase	Nitrite Reductase	Ca 2+ ATPase	Catalase ($E_{ref.}$ = 3.45 μmol)
	μmol Cyt C red min^{-1} mg^{-1}	*μmol Cyt C ox min^{-1} mg^{-1}*	*nmol NO^{-}_{2} red min^{-1} mg^{-1}*	*μmol Pi h^{-1} mg^{-1}*	*μmol H_2O_2 dec min^{-1}*
Root					
Plastids	0.0296	0.0156	93.310	0.04875	0.0276
Mitochondria	au[a]	0.4116	30.890	0.01623	0.0690
Microsomes	0.3300	au	au	0.08125	0.0759
Plasma membranes	au	au	au	0.22100	au
Leaf					
Chloroplasts	0.0222	0.0254	98.590	0.05177	0.0414
Mitochondria	0.0222	0.2685	22.098	0.04719	0.0483
Plasma membranes	au	0.0137	au	0.25588	0.0449

[a] Activity undetectable.

Sequences; Nakai and Horton, 1999) and ExPASy (Expert Protein Analysis System), a proteomics server of the Swiss Institute of Bioinformatics, provided valuable information pertaining to sorting signals and common motifs within MIPS primary structures and upstream open reading frames (ORFs). PSORT analyses predict that all four MIPS primary structures are type II membrane proteins with most of the primary structure being associated with cytoplasm (Table III). A predicted conserved transmem-

Table III. *PSORT predicted locations for MIPS and some of its putative targeting peptides*

Amino Acid Sequence	Predicted Location (Percent Probability)
Primary Structures	
Phaseolus, Arabidopsis (chromosome 2), Arabidopsis (chromosome 4), and Arabidopsis (chromosome 5)	ER membrane (85), plasma membrane (44), chloroplast thylakoid membrane (21), and mitochondrial inner membrane (10)
CEDSLLAAPIILDLVLLAELSTR	Transmembrane region
Putative Targeting Peptides	
Phaseolus	
MCISRIIIKETKRKREVCHTCRPPLCFILLHFSFISEQTK	Mitochondrial intermembrane space (82), matrix (55), inner membrane (28), and outer membrane (28)
MCISRIIIKETKRKDCKREVCHTCREKI	Mitochondrial intermembrane space (81), and nucleus (72)
MRPPLCFILLHFSFLISEQTK	Chloroplast thylakoid (80), mitochondrial matrix (43), mitochondrial intermembrane (78), and microbody (64)
MNPAKNYNKRNQKEVKGKMRLRPPL-CFILLHFSFLISEQTK	Nucleus (58), mitochondrial matrix (10), and chloroplast thylakoid membrane (10)
MNPAKDCKSREVCHTCRPPLCFILLHFSFLISEQTK	Extracellular (37), ER membrane (10), ER lumen (10), and Golgi body (10)
Arabidopsis (Chromosome 2)	
MHLDFIQTKVQKWRKIFYHLLW	Microbody (64), cytosol (45), and mitochondrial matrix (10)
MSCPTTTTASSSSSLGCLRVCSQIYKET-CLHVNHATCLNPTSNYSLTFSNSTLLVV-LVLSGLLDWARTISIPPITSK-PATCPPSSVSRLYIHALVRRFEITHTTTKHKAFQNPEK	Chloroplast thylakoid membrane (82), stroma (75), thykaloid space (75), and mitochondri inner membrane (61)
MHLDFIQTKVQKWRKIFYHLLWCPTTT-TASSSSSLGCLRVCSQIYKETCLHVN-HATCLNPTSNYSLTFSNSTLLVVLVLS-GLDWARTISIPPITSKPATCPPSSVSRLYIHALVRRFEIT-HTTTKHKAFQNPEK	ER (85), microbody (49), plasma membrane (44), and chloroplast thylakoid membrane (11)
Arabidopsis (Chromosome 4)	
MHWTNRDHQPVKKHKTHTPKPIQIPRKTTKFSFSSAK	Nucleus (81), microbody (64), and mitochondrial matrix (10)
Arabidopsis (Chromosome 5)	
MIDTNQSRSLFDVTLTLTYCHMYEVAK	Mitochondrial intermembrane space (81), mitochondrial matrix (50), inner membrane (22), and cytoplasm (45)

brane motif (CEDSLLAAPIILDLVLLAELSTR), located approximately 68 amino acids from the carboxy terminus, is also predicted. Database entries for MIPS, in fact, reveal that this transmembrane motif is not only present, but is extraordinarily conserved in representatives from both the plant and animal kingdoms and in organisms as different as Brewer's yeast and human (*Homo sapiens*) despite the former presumption that MIPS is a cytosolic non-membrane-associated enzyme. The genomic sequences immediately 5′ of the translation start codons are not conserved in the one bean and three Arabidopsis MIPS genes examined (Fig. 4, A–D). A striking commonality in these upstream regions is, however, the presence of short ORFs that could potentially encode transit peptides capable of targeting MIPS isoforms to a variety of subcellular locations (Table III). The Phaseolus gene contains upstream ORFs interspersed with consensus RNA splice sites that predict five such peptides, each with a high probability of directing the enzyme to a different cellular compartment, including the nucleus, thylakoid membranes of chloroplast, and microbodies (Fig. 4A; Table III). Each Arabidopsis gene has a distinct set of predicted transit peptides. Chromosome 2 gene has three such sequences, the first targeting with highest probability to the microbody, the second to chloroplast (thylakoid membrane, stroma, and thylakoid space), and the third to the endoplasmic reticulum (Fig. 4B; Table III). Chromosome 4 MIPS gene has one targeting sequence for the nucleus and the microbody (Fig. 4C; Table III). Finally, the chromosome 5 MIPS gene has

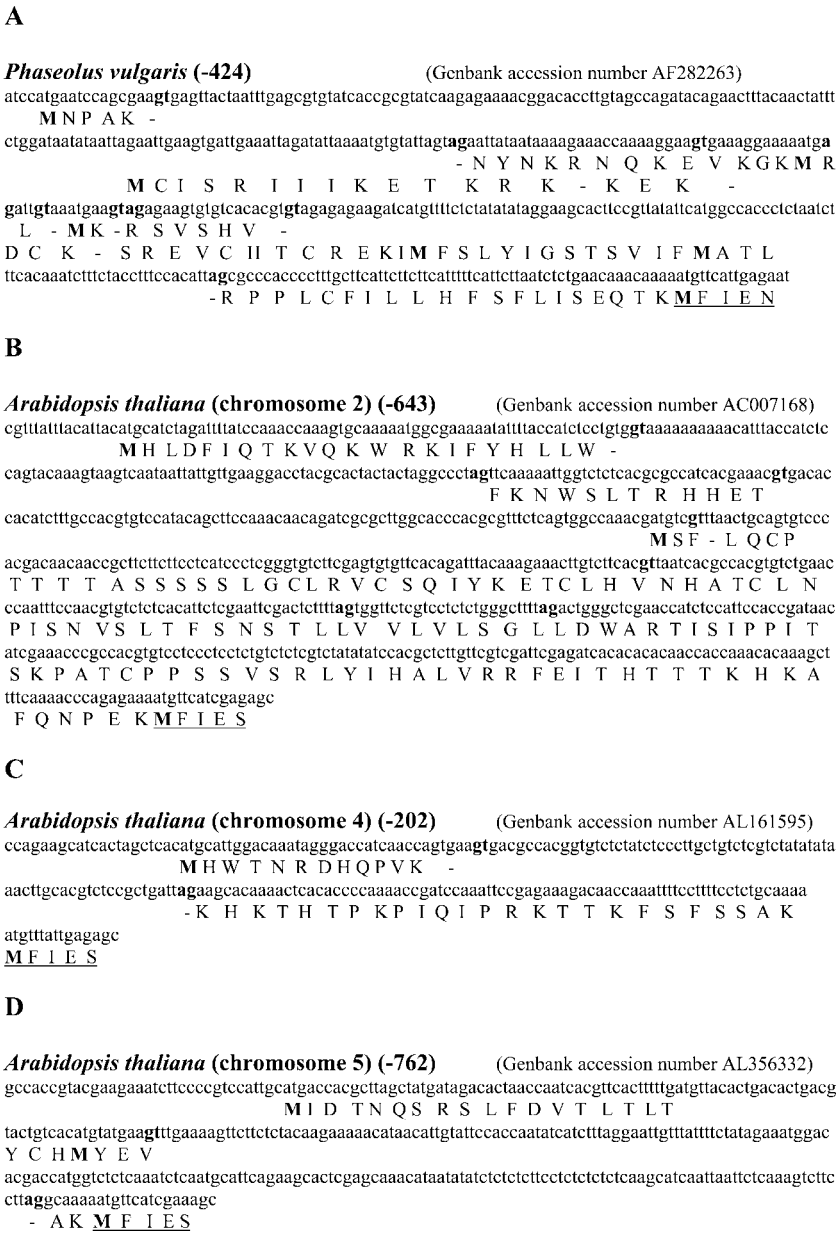

Figure 4. Computer analyses predict that MIPS and putative targeting peptides might localize to organelles identified as sites of inositol phosphate biosynthesis. Upstream regions of four MIPS genes (A–D) were analyzed for splice sites (nucleotides in bold type), Met residues (amino acids in bold type), stop codons, and organelle targeting ORFs. Negative numbers represent bases 5′ of published start sites (underlined amino acids). This information was used to generate putative targeting peptides for each gene (Table III). Bean MIPS is 90% identical to the Arabidopsis MIPS on chromosome 2, 96% identical to the one on chromosome 4, and 86% identical to MIPS on chromosome 5. PSORT (Table III) predicts the primary structures for all four MIPS can reside in the endoplasmic reticulum, plasma membrane, chloroplast thylakoid membrane, and mitochondria inner membrane. In addition, MIPS is predicted to be a type II membrane protein with most of its structure associated with the cytosol.

one such sequence with the most likely target being the mitochondrial intermembrane space (Fig. 4D; Table III). The ProfileScan computer program identified *N* glycosylation, protein kinase C phosphorylation, casein kinase II phosphorylation, and *N* myristoylation as possible posttranslational modifications for MIPS and some of its putative targeting peptides. Intriguingly, ProfileScan also ascribed a Wnt motif to bean putative transit peptide number 5 (Table III). Members of the Wnt family of secreted glycoproteins participate in many signaling events during development and play permissive roles during cell-fate assignment by interacting with a number of extracellular and cell-surface proteins (Arias et al., 1999).

Reverse Transcriptase (RT)-PCR

To systematically assess expression of the ORFs, the upstream region of the bean MIPS gene (Fig. 5B) was used to generate primers for RT-PCR experiments. Four forward primers (underlined sequence) and a reverse primer from within the MIPS coding sequence (third exon) were designed to detect appropriately spliced mRNAs that contain the upstream ORFs (Fig. 5, B–C). Sequencing of a leaf cDNA produced from primer 1 confirmed the existence of an appropriately spliced mRNA capable of producing peptide number 3 (Table III). Analysis of other products is currently in progress.

DISCUSSION

We have used a variety of experimental approaches including microscopic analyses, organelle isolations, western-blot analyses, and enzyme assays to question MIPS presence (i.e. inositol phosphate biosynthesis) in membrane-bound cellular compartments. Although MIPS has only been isolated in its soluble form, it is clear that other forms exist and that they are associated with membrane-bound organelles. Given the numerous cellular compartments and genetic loci for MIPS, it is reasonable to speculate that a probable function for the enzyme is to help regulate the complex metabolic flux of inositol. The identification of several new inositol-utilizing pathways within organelles (York et al., 1999; Irvine and Schell, 2001) suggests these pathways may draw from the same inositol pool, necessitating large quantities of

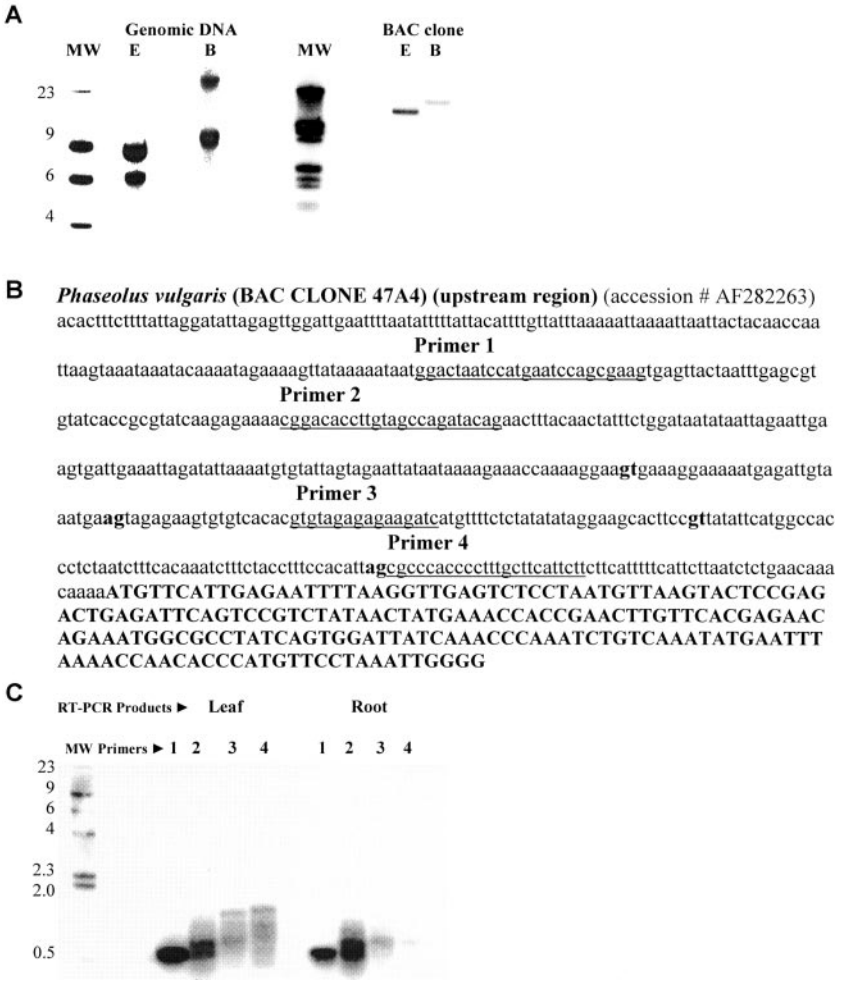

Figure 5. Phaseolus has two MIPS genes. One of two bean MIPS genes was isolated from a bacterial artificial chromosome (BAC) library, characterized, sequenced, and used to design primers for RT-PCR experiments. A, Southern-blot analysis of genomic DNA and BAC DNA digested with restriction enzymes not present in the gene sequence, *Eco*RI (E) and Bam HI (B), detected two hybridizing fragments (9 and 6 kb) in genomic DNA and one hybridizing fragment (9 kb) in the BAC DNA. The deduced amino acid sequence of the clone is 89% identical to the bean root cDNA used as probe for library screening (Wang and Johnson, 1995). These results suggest that there are two MIPS genes in the bean genome. B, Bean upstream region contains ORFs interspersed with consensus RNA splice sites (lowercase bold type) and the first exon (uppercase bold type). C, RT-PCR reactions were performed using a reverse primer (5′-CCTTGGCCC TACCCATGGC-3′) made from a sequence in the third exon and four different forward primers (underlined sequence). All lanes were loaded with RT-PCR reaction products (5 μL) generated from leaf or root mRNA.

readily available inositol phosphate that can easily be supplied and regulated at the level of inositol phosphate biosynthesis, i.e. MIPS. Likewise, a transmembrane orientation for MIPS places the enzyme in a pivotal position from which to regulate both membrane and non-membrane-bound aspects of the complex metabolic flux of inositol. It has been known for some time that MIPS is coordinately regulated with membrane-bound phospholipid biosynthetic enzymes in Brewer's yeast (Carmen and Henry, 1999) even though there is no available evidence that suggests that this regulation is mediated through the physical interaction of MIPS with these enzymes. Others have also found the expression of MIPS in membrane-bound cellular compartments. Wong et al. (1987) detected MIPS expression and activity in the walls of all the vascular elements including cerebral capillaries of bovine brain, whereas Yoshida et al. (1999) discovered MIPS transcripts in the scutellum and aleurone layers of rice embryos.

CONCLUSIONS

On the basis of these and other data (Lackey et al., 2002), we propose that a complex repertoire of cellular mechanisms functions to spatially and temporally control inositol phosphate biosynthesis during plant growth and development. Tagged Arabidopsis MIPS genes will be used to test this hypothesis and to question the role(s) of MIPS in inositol-signaling events.

MATERIALS AND METHODS

Plant and Yeast Growth Conditions

Bean (*Phaseolus vulgaris*) seeds (Taylor's horticultural variety) were purchased from Asgrow Seed Co. (Kalamazoo, MI) and were grown aseptically in agar medium containing a Murashige and Skoog salt base without inositol (Sigma-Aldrich, St. Louis) in an environmental chamber maintained at 24°C with 16-h photoperiods. Wild-type Brewer's yeast (*Saccharomyces cerevisiae*) strain SH 477 (Mat a, ura3), was grown at 30°C.

Protein Isolations and Western-Blot Analyses

Soluble plant and yeast proteins were isolated, and protein concentrations were determined as previously described, respectively (Johnson and Henry, 1989; Johnson and Wang, 1996). MIPS partial purification included the preparation of crude extracts, streptomycin sulfate precipitations, and ammonium sulfate fractionations (Johnson and Wang, 1996). Brewer's yeast polyclonal antibody to MIPS was used for western blotting as detailed previously (Johnson and Henry, 1989; Johnson and Wang, 1996).

Microscopic Analyses of Tissues Harvested Eight Days after Germination

For immunohistochemical studies, bean roots and leaves were fixed, embedded in paraffin blocks, sectioned on a New World 820 microtome, and stained with 1% (w/v) toluidine blue O (Sigma-Aldrich) to block autofluorescence. After overnight incubation with MIPS antibody (1:500 dilution), sections were incubated with goat anti-rabbit IgG (whole-molecule) fluorescein isothiocyanate-conjugated secondary antibody (1:100 dilution; Sigma-Aldrich), mounted in Mowiol (Calbiochem, San Diego) and photographed with a confocal microscope (PCM 2000, Nikon, Tokyo). Controls included unstained sections not treated with primary or secondary antibody to detect autofluorescence and stained sections incubated only with secondary antibody to detect nonspecific interactions.

Bean roots subjected to immunocytochemistry were fixed, embedded in Lowcryl K4M (Polysciences, Warrington, PA) as described (Altman et al., 1984), and transferred to capsules for polymerization over a 15-W black-light UV lamp at a distance of 10 cm for 45 min at 4°C. After sectioning, samples were placed on 300-mesh formvar coated nickel grids, blocked, incubated (first, with MIPS primary antibody [1:500 dilution] and then goat anti-rabbit 10-nm gold-conjugated secondary antibody [1:100 dilution] for 16 h at room temperature), viewed, and photographed with a microscope (10A TEM, Zeiss, Jena, Germany). Primary antibody was omitted from control grids to determine the amount of nonspecific binding by the gold-conjugated secondary antibody.

Isolation of Organelles from Eight-Day-Old Plants and Enzyme Assays

Plastids were isolated from roots using two successive Percoll gradients, rinsed, solubilized, and assayed for purity and enrichment (Robinson and Barnett, 1988). Chloroplasts were purified from leaves and cotyledons as detailed previously (Johnson and Wang, 1996). Mitochondria were harvested from green and non-green tissues using the procedures of Dounce et al. (1987) and Moore and Proudlove (1987), respectively. Mitochondrial fractions from roots were isolated from two sequential 28% (v/v) Percoll gradients, whereas fractions from green tissues were subjected to two successive discontinuous Percoll gradients. Purified mitochondria were washed, solublized, and assayed for purity and intactness.

Microsomes, endoplasmic reticula, and plasma membranes were collected as described (Surowy and Sussman, 1986). Purified organelles were dialyzed against gradient buffer and concentrated (concentrator solution, Pierce, Rockford, IL). Yeast microsomes, a negative control, were extracted (Carman and Fischl, 1992), washed, and assayed for MIPS expression and activity.

Catalase (EC 1.11.1.6) and cytochrome C oxidase (EC 1.9.3.1) activities were monitored as described (Johnson and Wang, 1996). Antimycin-insensitive NADH cytochrome C reductase (EC 1.6.99.3) activity (Briskin et al., 1987) and nitrite reductase (EC 1.7.7.1) activity (Wray and Fido, 1989) were assayed spectrophotometrically. Vanadate-sensitive Ca^{2+} ATPase (EC 3.6.1.38) activity was identified using the procedure of Surowy and Sussman (1986). MIPS (EC 5.5.1.4) activity was assayed by the end-product method (Chen and Charalampous, 1966) and the rapid colorimetric method (Barnett et al., 1970). D-[1-^{14}C]Glc 6-phosphate (specific activity 60.3 mCi mmol^{-1}) and [1,2-^{3}H]myoinositol (specific activity 370–740 GBq mmol^{-1}) were obtained from PerkinElmer Life Sciences (Boston). Glc 6-phosphate, bacterial alkaline phosphatase, and phosphate standard were purchased from Sigma-Aldrich.

Isolation of Phaseolus MIPS Genomic Clone and Expression Studies

A bean MIPS cDNA (Wang and Johnson, 1995) was used to isolate two genomic clones from an indexed BAC library. DNA sequencing of one clone, both strands, was performed at the Iowa State University DNA Sequencing Facility (Ames, IA). Southern-blot analyses (Sambrook et al., 1989) and RT-PCR (Promega, Madison, WI) were performed according to instructions.

Distribution of Materials

Upon request, all novel materials described in this publication will be made available in a timely manner for noncommercial research purposes, subject to the requisite permission from any third-party owners of all or parts of the material. Obtaining any permissions will be the responsibility of the requestor.

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